CXVII. OBSERVATIONS ON SOME ALCOHOL-SOLUBLE PROTEINS FROM MILK PRODUCTS.

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The presence of alcohol-soluble proteins in milk or cheese has been reported by several workers. Weidemann [1882] isolated from Emmenthal cheese by extraction with boiling ethyl alcohol a substance which he called caseoglutin. This was confirmed by Rose and Schulze [1884] and Benecke and Schulze [1887]. Winterstein and Thöni [1902], Winterstein [1904] and Bisegger [1907], also working with Emmenthal cheese, concluded that caseoglutin is formed during the ripening process. Nierenstein [1912], working with old Cheddar cheese, isolated from 6874 g. of cheese 82 g. of dried caseoglutin containing 14·52 % N. Osborne and Wakeman [1918] isolated from caseinogen prepared from milk a protein which was soluble in 50–70 % alcohol and very dilute acetic acid and gave a voluminous precipitate with potassium ferrocyanide. The tryptophan, Millon and biuret reactions were strongly positive. The phosphorus content was much less than that of caseinogen, and less arginine, histidine and lysine were found on hydrolysis. Anaphylactic reactions showed no genetic relationship between caseinogen and this alcohol-soluble protein. Grimmer and Wagenführ [1925] and Grimmer and Schutzler [1926] concluded from the analysis of the extract from Backstein cheese with 70 % alcohol that the caseoglutin obtained was a mixture of various substances, and that the composition was very variable.

Experimental.

In the course of the extraction of amino-acids from Cheddar cheese by the butyl alcohol method [Dakin, 1918] the presence of an alcohol-soluble protein was discovered.

To effect a preliminary separation of the amino-acids and lower polypeptides from caseinogen and the higher protein decomposition products in cheese 80 % alcohol was used. Powdered fat-free dried cheese was extracted with this reagent for 24 hours and the filtrate was evaporated at 40° under reduced pressure to obtain an aqueous residue for further extraction with butyl alcohol. It was found, however, that a bulky white precipitate was

formed as the ethyl alcohol was removed. The precipitate failed to dissolve on the addition of water to the aqueous residue but when ethyl alcohol was added until the concentration reached 80 % a clear solution was again obtained. A preliminary examination of the substance showed that it was a protein with a phosphorus content much less than that of caseinogen. A detailed investigation of its nature was therefore undertaken.

(*a*) *Protein from immature cheese.* An 18 weeks old Cheddar cheese was thoroughly grated and extracted with ether for 5 hours. The residue was dried in air for 24 hours at 55° to a dry, friable powder. 100 g. were suspended in 1 litre of 80 % alcohol and shaken at intervals over a period of 24 hours. The mixture was filtered and all the alcohol removed under reduced pressure at 40°. The bulky white precipitate, which was glutinous in parts, was filtered off and washed with cold water. On taking up in 80 % alcohol a slightly turbid solution was obtained. The turbidity was probably due to the denaturation of a portion of the protein since it had been previously noticed that the flocculent portion dissolved completely. On distilling this alcoholic solution at 40° under reduced pressure, filtering, washing the precipitate with water, and redissolving it in 80 % alcohol the amount of denaturation was found to be increased, with a lowering of the solubility.

The protein was precipitated from alcoholic solution with 2 volumes of ether, filtered and washed well with ether. This process was repeated once and the solid dried in air. In this way 0·8 g. of a pure white, friable powder was obtained.

Properties of the protein. The protein had the following properties.

(1) It was soluble in dilute alkali and re-precipitated from the solution on the gradual addition of dilute acid.

(2) An aqueous suspension dissolved on the addition of a little acetic acid.

(3) It was insoluble in water and absolute alcohol but completely soluble in 80 % alcohol. The colloidal suspension formed by adding water to this solution was flocculated by the addition of hydrochloric acid and could then be filtered.

(4) Sodium acetate gave no precipitate when added to the alcoholic solution. A fine white precipitate was produced by ether.

(5) Potassium ferrocyanide produced a bulky white precipitate from a solution in dilute acetic acid.

(6) The biuret, xanthoproteic and Millon reactions were positive and the Adamkiewicz and the Reichl reactions for tryptophan were also given.

(*b*) *Protein from mature cheese.* Investigations were made in order to see whether the same or a similar protein could be recovered from mature cheese. For this purpose a Cheddar cheese 16 months old, made from the same milk at the same time as the immature cheese, was used. 200 g. of fat-free, air-dried cheese were suspended for 24 hours in 1 litre of 80 % alcohol and then filtered. The filtrate was added to 3 volumes of water and the resulting precipitate separated by filtration. This light yellow precipitate failed to redissolve in

cold 80 % alcohol and became very glutinous. On warming to 40° some solution occurred, but separation into two layers took place on cooling, a slightly yellow, hard precipitate adhering to the bottom of the flask and a white, flocculent precipitate forming the top layer.

The white, flocculent precipitate was decanted and filtered, yielding a white, friable residue. Redissolving this residue in warm 80 % alcohol and adding the solution to two volumes of cold water produced a milky, colloidal suspension. On adding dilute alkali drop by drop a point was reached where the protein separated into flakes which filtered easily, leaving a clear filtrate of p_H 4·5. After drying in air at 30° the residue yielded 3·28 g. of a white, friable powder. The reactions were those of the protein isolated from the immature cheese.

The glutinous nature of the protein when mixed with alcohol appeared to be similar to that of the monocalcium paracaseinate of Van Slyke [1924] but its negligible ash content and insolubility in warm 5 % sodium chloride solution discounted this idea.

(*c*) *Protein from peptic digest of caseinogen.* In view of the presence in cheese of alcohol-soluble proteins an examination of the peptic digest of caseinogen for similar compounds was made.

100 g. of B.D.H. "Light White Casein" were extracted several times with 80 % alcohol at p_H 5·0, to remove any alcohol-soluble protein present, washed in ether and dried in air. Digestion with pepsin in 500 cc. of water acidified with hydrochloric acid was then carried out for 104 hours in the presence of chloroform. The volume was made up to 2½ litres with absolute alcohol, the mixture well shaken and the p_H adjusted to 5·0. Extraction was continued with occasional shaking for 15 hours and the solution filtered. When the alcohol was removed by distillation *in vacuo* at 40° a bulky precipitate separated. This was filtered, washed in ether, taken up three times in warm 80 % alcohol and finally dried. A yield of 0·25 g. of a white powder was obtained. The reactions were those of the first two proteins.

Composition of the three alcohol-soluble proteins. After drying *in vacuo* over sulphuric acid micro-combustions were carried out in duplicate on each protein to determine the carbon, hydrogen, nitrogen, sulphur, ash and moisture contents, by Dr Schoeller of Berlin. Table I shows the results of analysis.

Table I. *Composition of three alcohol-soluble proteins.*

Protein from ...	Cheese 4 months old	Cheese 16 months old	Peptic digest of caseinogen
C	52·43	52·04	52·51
H	7·98	7·55	7·26
N	14·36	14·15	14·38
S	0·93	0·83	1·02
Ash	0·003	Trace	0·004
Moisture	5·26	6·95	3·83

Phosphorus estimations. Colorimetric estimations by Briggs's [1922] method indicated a very low phosphorus content in all cases but by the method of

Benedict and Theis [1924], which avoids loss of phosphorus during incineration, appreciably higher values were obtained. For accurate determinations the method of Fiske and Subbarow [Hawk, 1927] and a dry ashing process were used. The method was tried out on a sample of caseinogen (prepared by the method of Van Slyke [1924]). Duplicate estimations gave values of 0·742 and 0·751 % for the phosphorus content of the moisture-free caseinogen. The values obtained for the phosphorus content of the three proteins were as follows:

Protein from ...	Cheese 4 months old	Cheese 16 months old	Peptic digest of caseinogen
P %	0·19	0·08	0·03

Discussion.

The composition of the three alcohol-soluble proteins, computed on a moisture- and ash-free basis, is shown in Table II, where the composition of caseinogen is also given for comparison.

Table II. *Composition of alcohol-soluble proteins and of caseinogen.*

Protein	Caseinogen	Cheese 4 months old	Cheese 16 months old	Peptic digest of caseinogen
C	53·11	55·34	55·93	54·60
H	7·05	7·68	7·23	7·03
N	15·65	15·16	15·21	14·95
S	0·82	0·98	0·89	1·06
P	0·82	0·20	0·09	0·03
O	22·55	20·64	20·65	22·33

It is evident that the proteins differ considerably in composition from caseinogen and show some appreciable differences between themselves. Their main characteristic is their very low phosphorus content. Reference to Table I shows that the ash content of each is negligible, so that it may be assumed that the phosphorus present is in organic combination and is not an inorganic contamination.

Although the three proteins exhibit very similar characteristics and each has a very low phosphorus content they are not identical in composition. It is therefore important to decide whether the proteins from the cheese were initially present in the milk from which the cheese was made or whether they are decomposition products of the casein. The protein isolated by Osborne and Wakeman [1918] from caseinogen prepared from milk is very similar to the three proteins discussed in this paper and has a low phosphorus content—0·08 %. However, the caseinogen used by these authors was prepared from milk by precipitation with dilute hydrochloric acid, the product being purified by dissolving in alkali and precipitating with acid several times. If, therefore, we regard the alcohol-soluble protein finally isolated from the caseinogen as being initially present in the milk, it must be assumed that such treatment is without effect on the caseinogen molecule.

Rimington and Kay [1926] found that during the action of pepsin on

caseinogen no measurable amount of free phosphoric acid was liberated. Trypsin, however, converted rather more than 50 % of the phosphorus into free phosphoric acid in 2 months, while 1 % alkali at 37° liberated all the phosphorus as phosphoric acid within 24 hours. Rimington [1927] isolated from a tryptic digest of caseinogen a phosphopeptone which contained 50 % of the organic phosphorus. The molecule of this compound contained nine amino-acid linkages and was split off from caseinogen with great rapidity. Posternak [1927] isolated three similar compounds from a tryptic digest of caseinogen, but Rimington suggested that the presence of a phosphorus-free compound which followed the precipitation of the phosphopeptone rather closely was responsible for the variety of compounds obtained by this author.

Several workers have recently claimed that caseinogen itself is not homogeneous but is a mixture of several different proteins. Kondo [1923–25] and Linderstrøm-Lang [1925–27] were led to this conclusion by the fact that the solubility of caseinogen in dilute hydrochloric acid was found to be a function of the amount of undissolved caseinogen and that by fractional precipitation fractions could be obtained which differed considerably in P/N ratios. Linderstrøm-Lang [1927–29], using 60 % alcohol as a differential slovent, obtained from caseinogen several fractions with very different phosphorus contents. By re-combining the fractions it was possible to secure a mixture which exhibited the same properties (*e.g.* specific rotation, solubility in 60 % alcohol, solubility in hydrochloric acid, rate of rennet coagulation) as the original caseinogen.

Svedberg and co-workers [1930], by the application of the sedimentation velocity method and the equilibrium method [Svedberg, 1926, 1927], found that caseinogen prepared by Hammarsten's method consisted of several proteins of different molecular weights. By extracting caseinogen with 70 % alcohol acidified with hydrochloric acid they obtained a protein which they found to be a definite chemical entity, with a molecular weight of 375,000. Caseinogen prepared by the method of Van Slyke [1924] was also found to be heterogeneous.

There is thus some evidence for the conclusion that caseinogen in the free condition is a mixture of several different proteins. In any method of preparation, however, the use of dilute acid and alkali is essential for the purposes of preparation and purification and, having regard to the fact that caseinogen has been shown to be easily acted upon by alkali and by trypsin, resulting in the removal of the phosphorus, it appears very probable that the caseinogen molecule is so delicately constituted and the linkages so fragile that the treatment with acid and alkali to which it is subjected in the course of preparation is sufficient to rupture the molecule in several places, giving rise to a mixture of proteoses of lower molecular weight and differing phosphorus contents.

On these grounds, therefore, it is considered that the two proteins isolated from cheese are probably mixtures of several similar compounds, resulting

from breakdown of the casein either by the rennin, bacterial enzymes, or possibly the action of acid. The attempt to obtain a similar alcohol-soluble protein from the peptic digestion of caseinogen resulted, it is true, in a yield of only 0·25 g. from 100 g. of caseinogen. However, the caseinogen used was first extracted several times with 80 % alcohol and it is possible that this treatment removed nearly all the alcohol-soluble protein which was capable of being formed from the caseinogen and that treatment of the remainder with pepsin resulted mainly in the formation of other and lower degradation products.

SUMMARY.

Three alcohol-soluble proteins—two from Cheddar cheese and one from a peptic digest of caseinogen—have been isolated and their properties studied. The proteins differ considerably from caseinogen in composition and have a very low phosphorus content. Evidence is cited for the view that they are decomposition products of the caseinogen or the casein.

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